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Mini Review

Leptospiral Immunoproteins as Potential Elements for Diagnostic Tests and Vaccine Development

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Abstract

Leptospirosis, which is caused by Leptospira spirochetes, is an acute, febrile, and systemic zoonotic infection that is particularly common in tropical regions around the world. The clinical manifestations range from an undifferentiated febrile illness to life threatening conditions. The majority (90%) of patients manifest a mild anicteric febrile illness, while minority of patients (%10) go on to develop a severe form that involves multiple organs and presents with high fever, jaundice, hemorrhagic diathesis, acute renal failure, hepatic necrosis, pulmonary involvement and cardiovascular collapse. In this paper, we have summarized past and current findings about leptospiral antigens that are conserved among pathogenic leptospires. We have also mentioned leptospiral immunoproteins being utilized for diagnostic tests and vaccine studies.

Introduction

Leptospirosis, caused by the pathogenic spirochete *Leptospira*, is an acute, febrile, and systemic zoonotic infection, which causes a large disease burden in tropical regions around the world. Leptospirosis in a common sense is probably the most common anthropo-zoonotic disease in the world but can only be detected when looked for. We detected 22 leptospirosis cases in just six months in a 1000 bed capacity tertiary care hospital serving in Istanbul, Turkey, despite no case has been reported in the same center for the past 20 years or more (1).

The clinical manifestations ranged from an undifferentiated febrile illness to life threatening conditions. The majority (90%) of patients presented with a mild anicteric febrile illness, while minority of the patients (10%) who went on to develop a severe form involving multiple organs demonstrated high fever, jaundice, hemorrhagic diathesis, acute renal failure, hepatic necrosis, pulmonary and cardiovascular involvement (2,3). This severe condition is known as Weil's disease. Mortality from severe forms of the disease is known to be between 5% and 40% (2-4). However, anicteric forms, which are seen in 90-95% of all leptospirosis patients, can also cause severe or significant clinical courses leading to important complications (1).

The severe form of leptospirosis (Weil`s disease) was first described in 1886 by a German Professor named Adolf Weil. He detected leptospirosis in 4 men who had severe jaundice, fever, hemorrhage with renal involvement in his studies and the disease Weil`s disease was named after him. During this period there were several other crucial researchers that achieved important scientific breakthroughs in the name of leptospirosis. Stimson discovered an agent in kidney tissue from a patient who died during a yellow fever epidemic in 1907. Seven years later in 1914, Japanese researchers under the direction of Ryokichi Inada discovered spirochetes in the liver of guinea pigs injected with blood from patients with Weil`s disease (5). The first leptospirosis cases in Turkey were reported by Dr. Nüzhet and Dr. Resit Riza in 1915 (6,7). Eventhough it's been 125 years since their first discovery, a truly effective and cross-protective leptospirosis vaccine is yet to be developed.

Leptospirosis mainly occurs after exposure to envi-

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ronmental sources, such as animal urine, contaminated soil or water (particularly during swimming), or infected animal tissue. Kidney involvement in many animal species is chronic in nature and results in the shedding of large numbers of leptospirae in the urine. Such reservoir animals living in highly populated areas are important in leptospirosis epidemiology. Portals of entry include cuts or abraded skin, mucous membranes, conjunctiva or even vaginal route. The infection is rarely acquired by ingestion of food contaminated with urine or via aerosols (2-4).

The infective agents of leptospirosis are slow growing anaerobic, finely coiled, thin and long spiral bacteria of the leptospira genus which includes 300 serotypes of pathogenic leptospires (8). Injuries resulting from the cork-screw-like burrowing motility of the organism may be important in hemorrhagic episodes and multiple organ involvement of the disease (9). On the basis of genetic analysis leptospires are divided into 20 genemospecies with pathogenic leptospires falling into 13 genetic types (8).

Laboratory diagnosis

In regions where scrub typhus and hantaviral infections are endemic, differentiation between scrub typhus, hantavirus infection, dengue fever, salmonellosis and haematological malignancies etc. and leptospirosis based on clinical criteria alone is not possible. So, specific laboratory tests are necessary to differentiate leptospirosis cases from some infectious and non-infectious diseases with similar clinical symptoms and findings. Cultivation or culture methods take too long to diagnose the disease. Therefore, serological and molecular diagnoses are crucial options for the initiation of effective antibiotic therapy and to prevent the development of the severe complications of acute leptospirosis such as hepatorenal failure, cardiac or respiratory insufficiency, aseptic meningitis or haemorrhagic diathesis.

Laboratory diagnosis depends on the typical two phase course of the disease. In the first week of clinical symptoms, when antibodies have not yet been produced, it is recommended to examine the material obtained from patients by molecular methods. During the second week of the disease, special serological methods as well as the Microscopic Agglutination Test (MAT) are used. Since MAT detects leptospirosis in the second week of the illness, more rapid and sensitive diagnostic methods are currently the research target of most studies. In many studies, diagnostic enzyme-linked immunosorbent assay (ELISA) kits for detecting IgM antibodies to confirm the acute form of leptospirosis are used. Whole-Leptospira-based serologic assays are commercially available in ELISA and other rapid formats, yet clinical evaluations have found that these assays have sensitivities of 28 to 72% during acute-phase illness (10, 11). Moreover, the sensitivity for these assays may be less than 25% for patients during the first week of critical illness (8, 9), when treatment with antibiotic therapy may be most effective.

MAT is serogroup specific but inadequate for rapid case identification since it can only be performed in a few reference laboratories and requires analysis of paired sera to achieve sufficient accuracy. Studies related to Leptospiral immunoproteins conducted in recent years have produced exciting results in diagnosis of the disease. In addition, vaccine studies on these leptospiral immunoproteins are also underway.

PCR-based detection methods have been developed, however their usage is limited to a number of reference laboratories. It is regrettable that although the major health burden of leptospirosis still exists in developing countries, PCR-based detection has not been implemented in these countries. In a recent study, Real-time PCR for detecting the gene encoding the superficial lipoprotein "LipL32" in the pre-analytic phase was shown to be a suitable, rapid and sufficiently reliable method for the diagnostics of the acute form of leptospirosis (8).

Further from the perspective of appropriate laboratory practice in the pre-analytic phase and prevention of false negative results, it is necessary to take biological samples before the commencement of antibiotic therapy or within 24 hours following antibiotic treatment. Leptospires are so sensitive to most antibiotics that they disintegrate quickly and are then devastated by the immune system. Results of the examination can be falsely negative in this case although leptospirosis is still developing in the patient (8).

Virulence factors expressed during host infection usually cause specific antibody responses and, accordingly, may serve as fresh markers for a recombinant protein-based serodiagnostic test. Surface-associated proteins, Leptospira immunoglobulin (Ig)-like proteins (LigA, LigB, and LigC), which have bacterial Ig-like (Big) tandem repeat domains found in virulence factors such as intimin of enteropathogenic Escherichia coli and in-

vasin of Yersinia pseudotuberculosis, have been identified as serodiagnostic markers (10). And furthermore, lig genes are present exclusively in pathogenic Leptospira species. In addition, they are expressed in virulent strains but not in strains that have been attenuated by culture passaging. Lig proteins are expressed during host infection and seem to induce strong antibody responses in patients.

Julia Croda et al. conducted a trial about inmunoglobulin like proteins as a serodiagnostic marker for acute leptospirosis. They confirmed that detection of rLigB (PCR) proteins is more sensitive than MAT and other ELISA test methods especially in acute phase of leptospirosis. The results which are acquired by the immunoblot assay suggest that it can be feasible to apply recombinant Lig protein fragments in improving high-performing lateral flow or dipstick assays. The improvement of such assays could be a progression in addressing the under-reporting of this overlooked illness and would allow the implementation of intervention strategies based on early case detection and timely initiation of antimicrobial therapy as a measure to prevent the progression of leptospirosis and its severe outcomes (10).

In an another study, Subathra et al. showed that recombinant OMPl-1 proteins based IgG ELISA appears to be a better alternative to MAT for the diagnosis of Leptospirosis (12). Further, Agampodi et al. conducted a trial and investigated the utility and limitations of direct multi-locus sequence typing (MLST) on qPCR positive blood to determine infective leptospira strains. They showed that MLST can be used to directly identify pathogenic Leptospira strains in blood samples obtained during acute illness without the need for culture isolation, yet it shows important limitations related to bacterial load (13).

Leptospirosis and Vaccination

There have been numerous researches on the vaccination of leptospirosis which include the following types of vaccination methods such as Killed whole cell vaccine, LPS based vaccines, protein antigen vaccines (OmpL1 and LipL41, LipL32/Hap-1, leptospiral Immunoglobulin-like proteins (Lig)) and cell mediated immunity.

1-Killed Whole Cell and Inactivated/Attenuated Vaccines

In the past, inactivated/killed and attenuated vaccines were always attempted. Currently available vaccines are the killed whole cell bacterins, widely used in animals but less in humans. It is generating mainly humoral immunity by stimulating antibody against LPS. Heat killed whole cell was used but their specificity is limited to serovar specificity. Certainly there are reports available with a limited period of cross protection but majority of studies are documented with serovar specific protection. Killed whole cell leptospiral vaccines are already available in several hyperendemic countries. Currently, there is a killed vaccine available in China, Japan, and Vietnam. As an example, the Japaneese leptospiral vaccine contains formalin killed leptospire. 500 million/ml of Copenhageni and 250 million/ml of Australis, Autumnalis,and Hebdomadis are the following concentrations and serovars.

Two subcutaneous injections of 1.0 ml given at a 7 day interval are used. The booster injection consists of 1.0 ml of vaccine given subcutaneously. The second initial dose is given within 5 years. The efficacy rates of whole cell vaccines are about 60-100% in certain populations. Local changeability in serovars of endemic leptospiral strains complicates the development of a vaccine that can be used worldwide (14). The side effects of this vaccine were reported as systemic and local reactions. The whole cell vaccine may trigger autoimmune diseases. The outer envelope vaccine studied in China showed better protection with less adverse effects and higher agglutinating titer than those in whole cell vaccine. Using outer envelope vaccine declined the number of the patients with vaccine-unrelated serogroup strains (14, 15, 16, 17, 18).

In humans the only licensed vaccine is an Inactivated leptospirosis vaccine which has been under production in Cuba since 2006 (19). The problems with inactivated leptospirosis vaccines are their short period of immunity, serovar specificities, and frequent (every 12 months) need of booster doses. In addition, there can be painful swelling after revaccinations (20). Another drawback with inactivated/attenuated/ killed whole cell vaccine usage is confusing diagnostic test results since they cause agglutinating antibodies. This means agglutinating antibodies can't be differentiated from antibodies produced by natural infections (21).

2-Lipopolysaccharides

Leptospire similar to other gram negative bacteria expresses lipopolysaccharide (LPS) and these could be an important candidate for the development of efficient vaccine. Among pathogenic Leptospires, one can

see differences in the composition of LPS that reflect the antigenic variations. Matsuo et al obtained three immunoreactive fractions by extracting the antigenic components from L.biflexa Patoc I (22). Without any side effects, administrating L.biflexa LPS preparation was protective against virulent L.interrogans serovar Manilae. (23). Immunization kept hamsters from bacteremia and prevented a renal carrier state (14, 24). During the repeating studies, identification of LPS fractions and LPS like substances (LPSLS) with antigenic potential and capability of protection were attempted. So, leptospira derived LPS seems to bear a promising immune potential (25).

3-Protein antigens:

The immunogenic proteins, especially OMPs may be effective as vaccinogens. Protein extracts prepared from a pathogenic Leptospira can induce protective immunity against heterologous serovar strain in an experimental animal model (15). These data point to the potential utilization of leptospiral proteins as candidates for a new vaccine that could induce good protection against diverse serovars. Subunit vaccines may also have fewer side effects than killed whole cell vaccine. Most important immunogenic proteins are explained briefly here below.

a) OmpL1 and LipL41:

The difference between OmpL1 and Lip L 41 is that the former is an outer membrane lipoprotein whereas the latter is a transmembrane protein. The similarity between them is they are both surface exposed. It should be noted that these proteins synergically have good immunoprotective effect in hamster models, while none of them has immunoprotective effect separately. Within the inclusion those two proteins a synergistic mechanism emerges and therefore this mechanism requires a comprehensive study as to be deeply analyzed. In this case, a recommendation can be given obstructing Omp1 and Lip41. Another learning point in this study is that in inducing immunoprotection, immunization based on membrane associated Omp1 and LipL41 was crucial and necessary (26, 27).

b) LipL32(Hap-1):

A 32 kDa protein was defined to be a dominant antigen with the best serodiagnostic use (28). This antigen was identified as LipL32, a major leptospiral outer membrane protein whose expression is restricted to pathogenic leptospira species (29). LipL32 is a 272 amino acid protein with a 19 residue lipidation signal sequence. The protein was confirmed to be lapidated through the incorporation of tritiated palmitate (28). LipL32 which is an outer membrane lipoprotein, one of the most abundant proteins in Leptospira and is equivalent to 75% of OMP of leptospirosis. This protein is also known as haemolysis associated protein-1 (Hap-1). This lipoprotein has been preserved in various pathogenic leptospires both on genetical and immunological terms. Additionally, most of the vaccination trials have been conducted on this protein and previously thought to have promising results. Antibodies of human body are induced by LipL32 antigen. A good sensitivity and specificity are revealed in a recombinant LipL32 antigen if ELISA test has been used in detecting human leptospirosis IgG. In order to achieve this a vaccination that utilizes an adenovirus vector and encodes LipL32 gene is found to be useful. The LipL32gene obtained from L. interrogans serovar Autumnalis give protective immunity against a challenge caused by L.interrogans serovar Canicola. Some authors previously believed that all of these these findings demonstrate the main difference that either alone or in combination LipL32 protein has immunoprotective activity whereas OMPL1 is immunoprotective in the presence of LipL41 (14, 21, 30). During the leptospiral infection LipL32 is a dominant antigen in the humoral immune response, recognized in sera of more than 95% human leptospirosis patients by developed suitable serological tests like Leptospira LipL32 ELISA kits etc. Identification of immunodominant B- and T-cell combined epitopes in outer membrane lipoproteins LipL32 and LipL21 of Leptospira interrogans. (30). Similar findings makes the protein of great importance as a diagnostic tool and vaccine antigen. Maneewattch et al showed that passive protection of hamsters is possible using a LipL32 monoclonal antibody As a theoretical approach an antibody response to LipL32 should protect against leptospirosis, and it was clearly successful (31,32).

As we pointed out above, recent efforts to develop recombinant vaccines against leptospirosis have focused on OMPs. The most outstanding protein in leptospiral proteome is an outer membrane lipoprotein of 32 kDa, LipL32 (19). We want to emphasize that this protein can be evaluated as a promising antigen for the development of a multiserovar vaccine. LipL32 is expressed in all pathogenic Leptospira spp., and it is

highly conserved and not expressed in the saprophytic L. biflexa. This protein binds to extracellular matrix components, as indicated by in vitro assays (19, 20, 21, 33) and crystal structure analyses (19, 22, 37). Moreover, LipL32 is expressed during mammalian leptospiral infection (17,21, 37). Different immunization strategies that have been tested with LipL32 have shown some immune protection when administered with naked-DNA (19, 24), recombinant adenoviral (19, 25, 33), and Mycobacterium bovis BCG (19, 26, 33) delivery systems. Yet, LipL32 produced no protection by recombinant subunit protein vaccination with either a Freund or aluminium hydroxide adjuvant (19, 27, 33). In addition, The Escherichia coli heat-labile enterotoxin (LT), and its closely affiliated homologue Vibrio cholerae cholera toxin (CT), consists of one A subunit with ADP-ribosyltransferase activity linked to five B subunits (17). The B subunit of Escherichia coli heat-labile enterotoxin (LTB) is highly immunogenic upon systemic and mucosal immunizations. Its adjuvant activity has been showed with unrelated antigens, both coadministered and linked by chemical conjugation or genetic fusion, exhibiting no toxic effect (19, 33).

Current data shows us that despite the high immunogenicity of LipL32, the efficacy of LipL32 vaccines is unconvincing. But although studies usually showed either no protection or low levels of protection in some experimental trials (32), recent trials have showed us promising results about LipL32 that it may be the most likely recombinant vaccine candidate.

c) Leptospiral immunoglobulin like proteins

Lig proteins, which are punctuated by tandem repeats of about 90 aminoacids of bacterial Ig like domains are surface exposed OMPs. LigA is only comprised of Ig-like domains. On the other hand, LigB has an extra domain to reside at the C terminus which is unique only to itself. The bacterial immunoglobulin-like domains are detected in a variety of adhesion proteins of pathogenic bacteria. For instance, the intimin and invasion of Y.pseudotuberculosis can be given as examples of this sort. N terminal lipobox is presented in both LigA and LigB and LigA is lapidated when it is expressed in E.coli (14). When attenuated by culture of L.kirshneri and L.interrogans it was observed that expression of lig protein and lig m RNA diminished and they all remind us Lig proteins are closely related with virulence (14). Moreover, in a mouse model of Leptospirosis, Lig proteins have protective immunity not only with homologous serovar Manilae, but the heterogeneous serovar Icterohaemorrhagiae (32). These data shows that Lig proteins can induce protective immunity against the challenge with strains of different leptospiral serovars (14).

Full length Genome and Vaccine Development Studies

The ability to quickly determine full length genome sequences led to a new modality, Today. Serovar Lai 56601 and serovar Copenhageni Fiocruz L1-130 are published as the full length genome sequences of two strains of L.interrogans (34). For leptospiral vaccine analysis of strain Fiocruz L1-130 has been used to identify candidate antigens (35).

By computer analysis, genes containing exportation signal peptides, transmembrane domains, lipoprotein signatures and homologies to known surface proteins were chosen. To use immunoblotting with leptospirosis patient sera, a total of 206 genes had been predicted and 150 of them were expressed in E.coli, purified. In immunoblotting, a total of 16 proteins reacted with convalescent protein sera. 16 proteins shown in the study contained some previously identified antigens like LipL32 and Loa 22, but LipL41 and OmpL1 were not detected and this points out a possible weakness of the "reverse vaccinology" for analysis of some proteins. 4 of the 10 proteins tested were highly conserved among different pathogenic leptospires. There are still some ongoing studies but the protective activity of these proteins remains to be determined (14).

At present, the best level of protection afforded by a recombinant vaccine is seen with the recombinant leptospiral immunoglobulin-like protein LigA from L. interrogans (29, 36, 37). However, even after six full genome sequences, molecular trials of multiple strains and several vaccine studies have been reported, LipL32 is still the most likely recombinant vaccine candidate (18, 35). Grassmann et al. conducted a study about the protection against lethal leptospirosis after vaccination with LipL32 coupled or coadministered with the B Subunit of Escherichia coli Heat-Labile Enterotoxin and they demonstrated that rLipL32 coadministered or coupled with rLTB is highly immunogenic and prevents hamsters from lethal leptospirosis (19, 33).

In a recent trial, Banihasemi R. et al have investigated the effects of different adjuvants on the induction of humoral immunity in hamster model and tried to find the best formulation for Leptospira killed whole culture

vaccine and proved that alum (aliminum) and EDO2 (olive and additives) adjuvants have significant efficacy with leptospira vaccine (38).

Conclusion

In conclusion, leptospirosis is a worldwide infection and may lead to fatal complications. There is currently several methods used to diagnose the condition, however the most common are; MAT, ELISA method, and PCR method (leptospiral immunoproteins). It is seen that instead of MAT and ELISA methods, the real-time PCR for detecting the gene encoding especially the superficial lipoprotein LipL32 is more suitable and sufficiently reliable method for the diagnostics of the acute form of leptospirosis. Real time PCR could really be useful in the diagnosis of leptospirosis between the 2nd to 21st days of disease (39). Therefore, early diagnosis is crucial for the initiation of effective antibiotic therapy and the prevention of severe complications of acute leptospirosis such as hepatorenal failure, cardiac or respiratory insufficiency, aseptic meningitis or hemorrhagic diathesis. Finally, vaccine trials around the world are producing exciting results, but further research especially relating to leptospiral immunoproteins is still required to identify an ideal vaccine to provide protective immunity against leptospirosis.

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